Chemical synthesis and hepatic biotransformation of 3α,7α-dihydroxy-7β-methyl-24-nor-5β-cholan-23-oic acid, a 7-methyl derivative of norchenodeoxycholic acid: studies in the hamster

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Abstract A new bile acid analogue, 3α,7α-dihydroxy-7β-methyl-24-nor-5β-cholan-23-oic acid (7-Me-norCDCA) was synthesized from the methyl ester of norursodeoxycholic acid, and its hepatic biotransformation was defined in the hamster. To synthesize 7-Me-norCDCA, the 3α-hydroxyl group of methyl norursodeoxycholate was protected as the hemisuccinate, and the 7β-hydroxyl group was oxidized with CrO_3 to form the 7-ketone. A Grignard reaction with methyl magnesium iodide followed by alkaline hydrolysis gave 7-Me-norCDCA (>70% yield). The structure of the new compound was confirmed by proton magnetic resonance and mass spectrometry. After intraduodenal administration of the ^{14}C-labeled compound into the anesthetized biliary fistula hamster, it was rapidly and efficiently secreted into the bile; 80% of radioactivity was recovered in 2 h. After intravenous infusion, the compound was efficiently extracted by the liver and secreted into the bile (>75% in 3 h). Most (93%) of the biliary radioactivity was present in biotransformation products. The major biotransformation product (48.7 ± 6.0%) was a new compound, assigned the structure of 3α,5β,7α-trihydroxy-7β-methyl-24-nor-5β-cholan-23-oic acid (5β-hydroxy-7-Me-norCDCA). In addition, conjugates of 7-Me-norCDCA with taurine (13.7 ± 5.0%), sulfate (10.3 ± 3.0%), or glucuronide (5.1 ± 1.7%) were formed. 7-Me-norCDCA was strongly choleretic in the hamster; during its intravenous infusion, bile flow increased 2 to 3 times above the basal level, and the calculated choleretic activity of the compound (and its metabolic products) was much greater than that of many natural bile acids, indicating that the compound induced hypercholeresis. It is concluded that the biotransformation and physiological properties of 7-Me-norCDCA closely resemble those of norCDCA. Based on previous studies, the major biological effect of the 7-methyl group in 7-Me-norCDCA is to prevent its bacterial 7-dehydroxylation in the distal intestine.—**Yoshii, M., E. H. Mosbach, C. D. Schteingart, L. R. Hagey, A. F. Hofmann, B. I. Cohen, and C. K. McSherry.** Chemical synthesis and hepatic biotransformation of 3α,7α-dihydroxy-7β-methyl-24-nor-5β-cholan-23-oic acid, a 7-methyl derivative of norchenodeoxycholic acid: studies in the hamster. *J. Lipid Res.* 1991. **32:** 1729–1740.

Supplementary key words bile acid analogue • Grignard reaction • 7-Me-norCDCA • taurine conjugate • glucuronide • sulfate • 5β-hydroxylation • hamster • choleresis • enterohepatic • *Mesocricetus auratus*

In recent years, we have synthesized and studied new bile acid analogues in order to develop agents that might be more effective in preventing and/or dissolving cholesterol gallstones than the naturally occurring bile acids, chenodeoxycholic acid (CDCA) and ursodeoxycholic acid (UDCA). For this purpose, 7-methyl- and 6-methyl-substituted dihydroxy bile acids were synthesized (1–3) and their metabolism and cholelitholytic effect were characterized in animal models (3–6). The bile acid analogues examined so far were well absorbed from the intestine, were efficiently conjugated with glycine and taurine during hepatic transport, and were rapidly secreted into bile. The conjugates were absorbed from the ileum, like natural conjugated bile acids, and underwent enterohepatic cycling. The conjugates were deconjugated by intestinal bacteria, but in contrast to natural primary bile acids, neither the 7-methyl- nor the 6-methyl-substituted bile acids underwent bacterial 7-dehydroxylation to form the corresponding methyl-substituted lithocholic acid analogues (3–5). Not only were these compounds not dehydroxylated, but administration of 7β-methyl-cholic acid (7-Me-CA) or 7β-methyl-chenodeoxycholic acid (7-Me-CDCA) in the diet prevented the bacterial 7-dehydrox-

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Abbreviations: 7-Me-norCDCA, 3α,7α-dihydroxy-7β-methyl-24-nor-5β-cholan-23-oic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; CA, cholic acid; UDCA, ursodeoxycholic acid; HPLC, high pressure liquid chromatography; PMR, proton magnetic resonance; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; 7-Me-CA, 7β-methyl-cholic acid; 7-Me-CDCA, 7β-methyl-chenodeoxycholic acid; 6-Me-HDCA, 6-methyl-substituted hyodeoxycholic acid; 6-Me-MDCA, 6-methyl-substituted murideoxycholic acid; TMS, trimethylsilyl.

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ylation of the primary bile acids, cholic acid (CA) and CDCA, possibly via their antibacterial action (7, 8). Recently, we reported that the formation of cholesterol gallstones in the prairie dog was inhibited by 7-Me-CDCA as effectively as by CDCA, while 7-Me-CA was not cholelitholytic (8). We further found that 6-methyl-substituted hyodeoxycholic acid (6-Me-HDCA) and murideoxycholic acid (6-Me-MDCA) prevented the formation of cholesterol gallstones in a new hamster model of cholesterol cholelithiasis (6).

The physiological properties of the 23-nor derivatives of several of the common natural bile acids (norCa, norCDCA, and norUDCA) have been investigated during this time period at the University of California San Diego (9-12). These bile acid homologues have an isobutyric acid side chain instead of the isopentanoic acid side chain of the naturally occurring bile acids. When these norbile acids were infused into laboratory animals, they underwent little aminoacyl amidation with glycine or taurine. Instead, they were recovered in bile in the unconjugated form as well as in the form of conjugates with glucuronate or sulfate (9-15). The intravenous infusion of dihydroxy norbile acids produced a pronounced bicarbonate-rich choleresis called "hypercholeresis," which was thought to be caused by cholehepatic shunting of the unchanged, unconjugated molecule (9-12, 16-19).

In this study, we describe the synthesis of the 7-methyl derivative of norCDCA and the characterization of its hepatic metabolism in the biliary fistula hamster. The hypothesis to be tested was that the greater hydrophobicity of the 7-methyl compound would cause it to have different biological properties than norCDCA.

MATERIALS AND METHODS

General

Melting points were determined on a Thermolyne melting point apparatus and are not corrected. Radioactivity was determined in a Beckman LS 3801 liquid scintillation system (Beckman Instruments, Fullerton, CA) with automatic quench correction, using Aquasol-2 (NEN Products, Boston, MA) as the scintillator.

Proton magnetic resonance (PMR) spectrometry

Proton magnetic resonance (PMR) spectra were recorded on either a JEOL (Peabody, MA) GX-270 spectrometer at 270 MHz or a JEOL JNM-PS-100 spectrometer in pyridine-d_5 solution at 100 MHz (for the synthetic compounds), or a 360 MHz instrument equipped with a modified Varian HR-220 console, an Oxford magnet, and a Nicolet 1180-E computer system. Chemical shifts are given in ppm relative to tetramethylsilane (0.00 ppm) as an internal standard.

Chromatography

Thin-layer chromatography (TLC) was performed on precoated silica gel 60 F_{254} plates (0.2 mm thickness, EM Science, Darmstadt, Germany) using a solution of phosphomolybdic acid in ethanol, 10 g/dl to detect the spots. Preparative TLC was done on precoated silica gel G plates (20 × 20 cm, 0.25 mm thickness, Analtech Inc., Newark, DE). Solvent system A was chloroform-methanol-acetic acid-water 65:20:10:5 (v/v/v/v); solvent system B was isopropanol-benzene-acetic acid 10:30:0.1 (v/v/v). Silica gel 60 (35-70 mesh) from EM Science was used for preparative column adsorption chromatography.

Gas-liquid chromatography (GLC) was carried out on a Hewlett-Packard 5830A gas chromatograph using 3% SP-2250 and/or 3% Poly I-110 columns on 80/100 mesh Gaschrom Q (Supelco, Bellefonte, PA). The bile acids were analyzed as their methyl ester trimethylsilyl (TMS) ether derivatives. All retention times are reported relative to that of the TMS ether of methyl cholate (1.00). Gas-liquid chromatography-mass spectrometry (GLC-MS) was carried out on a Hewlett-Packard 5992B spectrometer under the following conditions: column, 3% SP-2250; column temperature, 260°C; injection port temperature, 265°C; source pressure, 2×10^{-6} torr; electron energy, 70 eV. For high-pressure liquid chromatography (HPLC) a Varian model 5000 liquid chromatograph was used; the instrument was equipped with a variable wavelength UV detector, Waters 481 spectrometer (Waters Associates, Milford, MA). The bile acid analogues were analyzed as their methyl ester derivatives. The analytical conditions were Radial-Pak C_{18} cartridge (Waters Associates) in a Waters Z-module; solvent, methanol-water 8:2 (v/v); flow rate, 2 ml/min; detector set at 203 nm.

Synthesis of 7-Me-norCDCA (III, Fig. 1)

Methyl 3α-succinoxy-7-oxo-24-nor-5β-cholan-23-oate (**II**). Methyl 3α-succinoxy-7-oxo-24-nor-5β-cholan-23-oate (II) was prepared according to the method of Hsia (20).

Methyl norursodeoxycholate (**I**, Fig. 1) (0.6 g) was refluxed with succinic anhydride (2.0 g) in dry benzene (50 ml) for 48 h and the solvent was removed under vacuum. The residue was dissolved in acetic acid (50 ml), and a solution of CrO_3 (1 g) in 80% acetic acid (2.0 ml) was added dropwise; the reaction mixture was then allowed to stand for 44 h at room temperature. The mixture was poured into ice water, and the resultant precipitate was crystallized from methanol-water. Methyl 3α-succinoxy-7-oxo-24-nor-5β-cholan-23-oate (**II**) (195 mg) was obtained in the form of colorless needles, mp 161-164°C. TLC showed an R_f value of 0.28 (solvent system B), PMR 0.59 (3H, s, 18-CH_3), 1.05 (3H, d, J=6.41,

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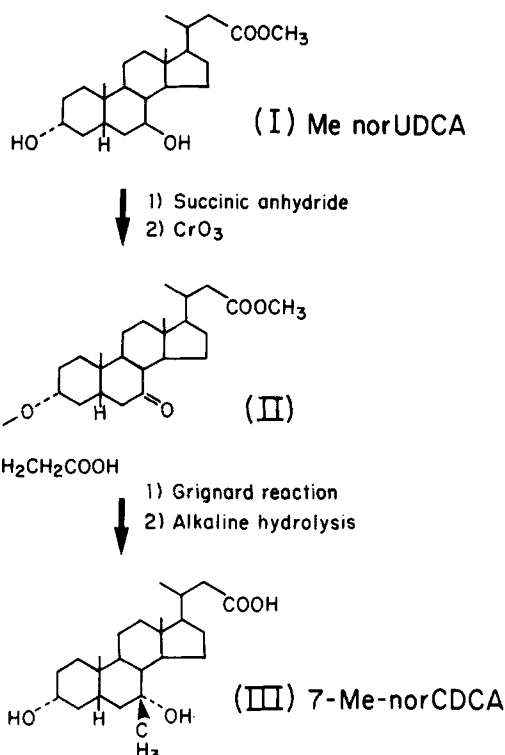

Fig. 1. Synthesis of the bile acid analogue, 3α,7α-dihydroxy-7β-methyl-24-nor-5β-cholanoic acid. I. Methyl 3α,7β-dihydroxy-24-nor-5β-cholanoate; II. Methyl 3α-succinoxy-7-oxo-24-nor-5β-cholanoate; III. 3α,7α-Dihydroxy-7β-methyl-24-nor-5β-cholanoic acid (7-Me-norCDCA).

21-CH_3), 1.08 (3H, s, 19-CH_3), 3.68 (3H, s, -$COOCH_3$), 4.82–4.89 (1H, m, 3β-H).

7-Me-norCDCA (III). To methyl 3α-succinoxy-7-oxo-24-nor-5β-cholan-23-oate (**II**) (1.0 g; 1.97 mmol) in anhydrous benzene (40 ml) 7.8 mmol CH_3MgI in 2.6 ml of anhydrous diethyl ether was added. The reaction mixture was stirred for 1 h at room temperature. Excess reagent was destroyed with 1 N HCl (20 ml), and the crude product was extracted with ethyl acetate. Evaporation gave a solid residue that was hydrolyzed with 5% methanolic KOH (50 ml). After acidification and extraction with ethyl acetate, a colorless residue was obtained that crystallized from ethyl acetate. The residue was recrystallized twice from ethyl acetate giving colorless needles of 7-Me-norCDCA (**III**). Yield 345 mg; mp 232.0–234.0°C, R_f 0.27 (solvent system B), RRT on GLC 0.93 (Poly I-110, methyl cholate = 1.00), PMR: 0.722 (3H, s, 18-CH_3), 0.872 (3H, s, 19-CH_3), 0.994 (3H, d, J=6.1 Hz, 21-CH_3), 1.225 (3H, s, 7β-CH_3), 2.179 (1H, q, J=12Hz, 4α-H), 2.434 (1H, q, J_1=14.3 Hz, J_2=2.9 Hz, 22-CH_2), 3.493 (1H, sep, 3β-H). GC-MS, 550, M^+[10.7]; 535, M−15[16.1]; 445, M−(90+15)[7.7]; 370, M−2×90 [14.7]; 257, C3-7 [100]; 143, C5-7 [98.6]. [The Grignard reaction gave 72.6% 7-Me-norCDCA as the major product, 19.7% was 7-methyl-24-nor-5β-cholane-3α,7α,23-triol, and 7.7% was starting material.]

Labeled compounds

[7β-methyl-^{14}C]7β-Me-norCDCA (sp act 3.0×10^5 dpm/mg) was prepared as described above using [^{14}C]CH_3MgI (prepared from [^{14}C]methyl iodide, 1.0 mCi, sp act 11.3 mCi/mmol, NEN Products). Radiochemical purity of the labeled compound was better than 92% as determined by radio-TLC. The labeled compound was dissolved in a 1% aqueous $NaHCO_3$ solution (concentration 2.7 mg/ml).

[11,12-^{3}H]norCDCA was prepared and purified as described previously (11).

Animal experiments

Male golden Syrian hamster (Charles River Breeding Laboratories, Wilmington, MA), weighing 100–140 g, were maintained for at least 2 weeks on a commercial rodent chow. The animals were given food and water ad libitum, and were kept under a controlled light/dark cycle. All animals were operated on between 9 and 10 AM. The animals were anesthetized by intraperitoneal injection of ketamine (Ketaset, Bristol Labs, Syracuse, NY); dose 10 mg/100 g body wt. Intramuscular injections of pentobarbital (Nembutal sodium solution, Abbott Labs, North Chicago, IL) were used to maintain anesthesia as required.

A polyethylene catheter (PE-10, 0.28 mm ID and 0.61 mm OD, Clay-Adams, Parsippany, NJ) was inserted into the left femoral vein and 0.9% NaCl solution was infused at a rate of 1.1 ml/h using a Harvard syringe (Millis, MA). The abdomen was opened by a midline incision, the cystic duct was ligated with a plastic clip (Absolok MCA, Ethicon Inc., Somerville, NJ), and an external biliary fistula was constructed using PE-10 tubing. The urethra was ligated to allow urine to accumulate in the bladder.

In the intravenous infusion studies, saline was infused into the femoral vein for 20 min prior to the administration of the labeled compounds. Each labeled compound was then infused for 20 min at a dose of 50 μg/min (1.27 μmol/kg·min) [total amount administered, 1 mg (2.55 μmol) (5)]; saline was then again infused until the end of the experiment. Bile samples were collected in tared tubes every 20 min for a total period of 4.0 h. At the end of the infusion, blood and urine were obtained by cardiac puncture and by aspiration from the urinary bladder, respectively, and the liver was excised. All biological specimens were stored at −20°C. In control experiments, saline was infused throughout the experimental period. In intraduodenal experiments, after cannulation of both the bile duct and femoral vein as described above, the labeled compound (500 μg/μmol) was injected into the duodenum. In the choleresis experiment, the bile acid was infused at a rate of 1 μmol/kg·min for 2 h. Bile samples were collected every 20 min for 3.5 h.

To define the biotransformation of the natural bile acid, CDCA, in the hamster, a single bolus dose of 8 μmol was

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injected into the perfusate of an isolated perfused hamster liver. Bile samples were obtained at 15-min intervals and the sample obtained at 30 min was analyzed by TLC. With the preparation of the isolated perfused liver used for this experiments, this dose corresponds approximately to 1 μmol/min · kg, a dose in the physiological range (12).

Analytical techniques

In the biotransformation experiments, bile samples were collected every 20 min. Four aliquots were taken from each bile sample to determine the metabolite(s) of the administered labeled compound as follows. *1*) The radioactivity of the first set of aliquots was determined by liquid scintillation counting. *2*) The second set of aliquots was analyzed by TLC to check the conjugation pattern using solvent system A, chloroform-methanol-acetic acid-water 65:20:10:5 (v/v/v/v). Four μl of bile was applied directly to a TLC plate along with reference compounds. After development (16 cm) and detection of the spots, each TLC plate was cut into 5-mm segments from the origin to the solvent front and the radioactivity of each segment was measured as described previously (5). *3*) The third set of aliquots was taken from bile collected during the fourth collection period (60 to 80 min) which contained the highest radioactivity in each animal experiment; these samples were hydrolyzed with cholylglycine hydrolase (EC 3.5.1.24; Sigma) (5) and β-glucuronidase (EC 3.2.1.31, Type H-1, *Helix pomatia*; Sigma, St. Louis, MO) (21) followed by solvolysis (22). The bile salts were extracted and analyzed by TLC using solvent system B, isopropanol-benzene-acetic acid, 10:30:0.1 (v/v/v). Radioactivity was determined as described above.

To confirm biliary bile acid composition, hydrolyzed and solvolyzed bile salts were also applied on preparative silica gel G plates and developed in solvent system B. After development, the plates were air-dried, exposed to iodine vapor, and the pertinent bands that contained radioactivity were scraped off. Bile acids were recovered by extraction with methanol and analyzed by GLC. *4*) A fourth aliquot (10 μl from each bile sample) was analyzed directly for bile acids by GLC (as methyl ester-TMS ether derivatives after enzymatic hydrolysis and solvolysis). Relative retention times were: deoxycholic acid (DCA) 1.07; CDCA 1.07; CA 1.00; 7-Me-norCDCA 1.48 on SP-2250; and DCA 1.28; CDCA 1.42; CA 1.00; 7-Me-norCDCA 1.14; and the hydroxylated metabolite of 7-Me-norCDCA 0.93, on Poly I-110.

To identify the major polar metabolite of 7-Me-norCDCA, bile collections containing most of the administered radioactivity were pooled. The bile acids were extracted, methyl-esterified, and purified by silica gel column chromatography. Two columns were used; the first was eluted with increasing amounts of acetone in benzene, the second with chloroform-methanol. 7-Me-5β-hydroxy-norCDCA was eluted from the first column with 22.5% acetone in benzene and, from the second column, with 10% methanol in chloroform.

Aliquots of urine and serum were checked for radioactivity. Bile salts in the liver were analyzed according to the method of Yanagisawa et al. (23) and their radioactivity was measured as described above.

Calculations

The numerical data are expressed as means ± SEM.

RESULTS

Metabolism of 7-Me-norCDCA in the anesthetized biliary fistula hamster

^{14}C-labeled 7-Me-norCDCA was infused intravenously or injected intraduodenally into bile fistula hamsters. **Table 1** summarizes the fate of the administered label in bile, urine, liver, and blood. In the 4-h collection period, more than 75% of the administered radioactivity was secreted into the bile and less than 0.5% was excreted into the urine. Radioactivity in the liver accounted for about 6%; the radioactivity in blood was too low to be detected.

Fig. 2 shows the cumulative recovery of the radioactivity secreted in bile following intravenous administration

TABLE 1. Fate of radioactivity after intravenous (iv) or intraduodenal (id) administration of ^{14}C-labeled 7-Me-norCDCA into hamsters

Number of Animals	Administration	Isotopic Recovery				
		Bile	Urine	Liver	Blood	Total
				%		
3	iv	76.4 ± 7.9[a]	0.4 ± 0.1	6.1 ± 3.9	N.D.[b]	82.9 ± 13.1
3	id	78.5 ± 10.5	0.1 ± 0.1	5.6 ± 5.0	N.D.[b]	84.2 ± 14.1

The ^{14}C-labeled compound was infused intravenously (iv) into biliary fistula hamsters at a rate of 50 μg/min (1.27 μmol/kg · min) for 20 min. Intraduodenal (id) administration was via bolus injection of 500 μg (1.25 μmol). Bile was collected for 4 h, and urine, liver, and blood samples were then obtained to determine their radioactivity.
[a]The data are expressed as mean ± SEM.
[b]N.D., not detected.

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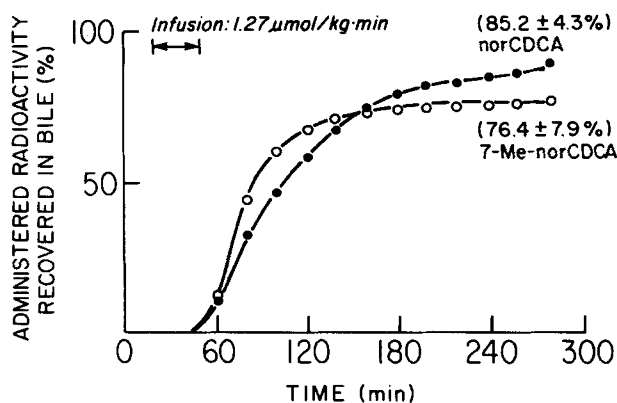

Fig. 2. Biliary recovery of radioactivity in bile fistula hamsters after intravenous infusion of ^{14}C-labeled 7-Me-norCDCA or [11,12-^{3}H]norCDCA. After a 40-min control period (saline), each bile acid was infused at a rate of 50 µg/min for 20 min.

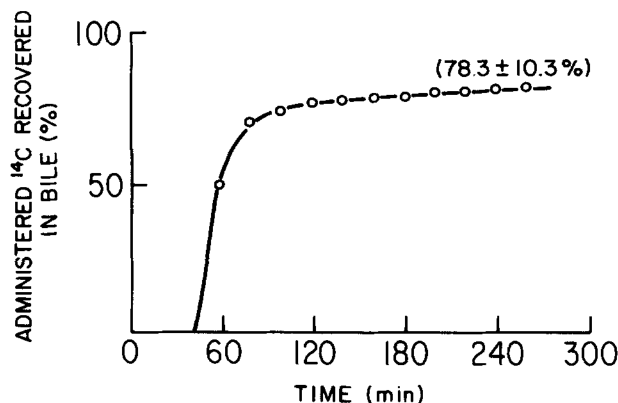

Fig. 3. Biliary recovery of radioactivity in bile fistula hamsters after intraduodenal administration of ^{14}C-labeled 7-Me-norCDCA. The bile acid was injected after a 40-min control period.

of [^{14}C-7β-methyl]7-Me-norCDCA. Radioactivity appeared in the bile within 20 min and more than 70% was recovered within 2 h. From the data of Figs. 2 and 3 it can be calculated that the half times ($t_{1/2}$) of biliary recovery were 45 min (intravenous infusion) and 19 min (intraduodenal injection). For comparison, [11,12-^{3}H]norCDCA was infused intravenously into three additional hamsters, under identical conditions (Fig. 2). The time course of the recovery of norCDCA radioactivity in bile closely resembled that of the 7-methyl analogue.

Fig. 3 illustrates the time course recovery of radioactivity in bile after intraduodenal injection of [^{14}C]7-Me-norCDCA. The data show that within 2 h nearly 80% of the administered dose was absorbed from the intestine and secreted into bile.

Biotransformation of 7-Me-norCDCA

The bile fractions from each animal containing the highest radioactivity were analyzed by radio-TLC in an attempt to elucidate the structures of the biotransformation products of 7-Me-norCDCA.

Fig. 4 shows the qualitative analysis by radio-TLC of the bile collected after intravenous infusion of [^{14}C]7-Me-norCDCA, and **Table 2** illustrates the quantitative analysis of the labeled metabolites following intravenous infusion or intraduodenal injection. The data show that only small amounts of the [^{14}C]7-Me-norCDCA administered by the two routes remained unchanged (about 7%); the remainder was transformed into more polar compounds due to either hydroxylation, sulfation, glucuronidation, and/or aminoacyl amidation with taurine.

The hydroxylated metabolite of 7-Me-norCDCA had an R_f value similar to that of α-muricholic acid (solvent systems A and B) and amounted to about 50% of biliary radioactivity, whereas taurine-conjugated 7-Me-norCDCA accounted for only 14–19%. (Conjugation with glycine was too low to be detectable under the conditions employed, <1.5%.) 7-Me-norCDCA sulfate accounted for 6–10%, and glucuronides of 7-Me-norCDCA for 3–5% of the radioactivity recovered in bile (Table 2).

Under similar conditions, about 40% of norCDCA infused intravenously was converted to the 5β-hydroxy metabolite; 17% was secreted unchanged and 18% was found to be conjugated with taurine, sulfate, or glucuronate (Table 2). Table 2 also includes data on the biotransformation of the natural C_{24} homologue, CDCA, in the

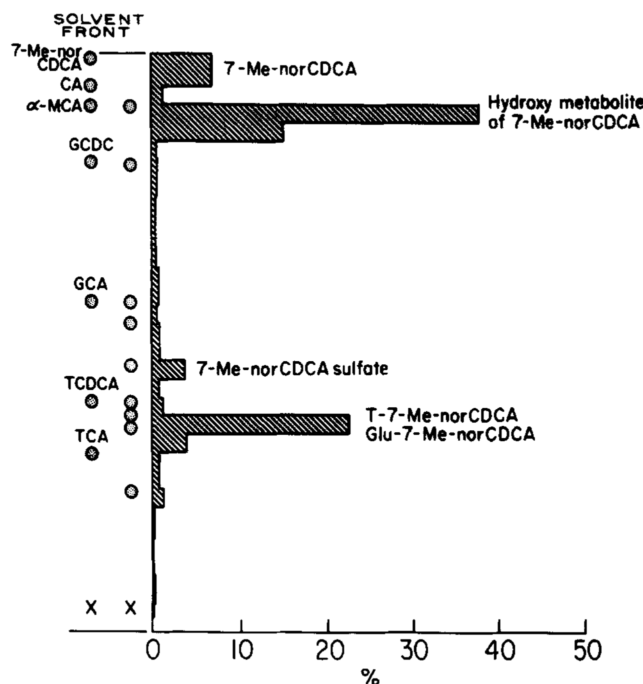

Fig. 4. Thin-layer chromatographic analysis of radioactivity recovered in bile of a bile fistula hamster infused with labeled 7-Me-norCDCA. The bile sample was analyzed by TLC (solvent system A, see Methods) before hydrolysis or solvolysis. Reference compounds are as follows: TCA, taurocholate; TCDCA, taurochenodeoxycholate; GCA, glycocholate; GCDCA, glycochenodeoxycholate; CA, cholic acid; α-MCA, α-muricholic acid; 7-Me-norCDCA, 7-methyl-norchenodeoxycholic acid.

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TABLE 2. Biotransformation profile of 7-Me-norCDCA after intravenous (iv) or intraduodenal (id) administration into bile fistula hamsters[a]

Compound Administered	Number of Animals	Biotransformation Profile: Hydroxylated	Unchanged	Conjugates: Amino Acylamidates: Taurine	Glycine	Sulfate	Glucuronide(s)[b]
				%			
7-Me-norCDCA (iv)	3	48.7 ± 6.0	6.6 ± 3.2	13.7 ± 5.0	0	10.3 ± 5.3	4.1 ± 2.0
NorCDCA (iv)	3	39.0 ± 1.8	16.5 ± 6.2	12.6 ± 3.7	0	2.7 ± 0.8	2.2 ± 1.2
7-Me-norCDCA (id)	3	51.3 ± 8.2	6.7 ± 2.3	19.3 ± 5.9	0	6.1 ± 1.1	3.0 ± 1.0
CDCA[c]	1	1.4	1.1	28.8[d]	59.5[e]	7.7	0.0

[a]Bile samples were analyzed by thin-layer chromatography using solvent system A (chloroform-methanol-acetic acid-water 65:20:10:5) followed by liquid scintillation counting.
[b]Using methods previously described from the UCSD laboratory (18), the glucuronide conjugates were found to be about half 23-ester conjugates and about half 3-ethereal conjugates.
[c]Bile samples were analyzed by thin-layer chromatography-zonal scanning (18).
[d]Includes 3.7% of a taurine-conjugated trihydroxy compound, presumably β-muricholate.
[e]Includes 11.3% of the 3-oxo derivative of the glycine conjugate, that is, 3-oxo-7α-hydroxy-5β-cholan-24-oyl-glycine.

isolated perfused hamster liver. The compound was mostly conjugated with glycine or taurine. Little nuclear hydroxylation occurred.

PMR spectrometry and mass spectrometry of the trihydroxy biotransformation product

The trihydroxy metabolite was isolated and purified by chromatographic methods and further examined by PMR and GLC-MS. **Table 3** lists the PMR spectra of the unknown and of the parent compound, 7-Me-norCDCA. The metabolite did not show any new signal due to an additional secondary alcohol, indicating that the starting material had been hydroxylated on the tertiary position. The changes in chemical shift and couplings of the signal, corresponding to H-4α, provided the key to the identification of the hydroxylation position.

In the starting material H-4α appears at 2.179 ppm as a quartet with J 12Hz. The unusual deshielding is due to the proximity of this proton to the 7α-hydroxyl group, which occurs only in 5β-steroids (24). H-4α is coupled to H-4β (geminal) and to H-3β and H-5 (axial-axial), with very similar large coupling constants, resulting in the observed quartet. In the trihydroxy metabolite, this signal appeared even more deshielded (2.496 ppm) as a double-doublet with two large coupling constants. This pointed to the disappearance of H-3, H-4β, or H-5. Since H-3 is still present as the usual "septet" and H-4α had not been deshielded to the 3-4 ppm region characteristic of secondary alcohols, the necessary conclusion was that the new hydroxyl group was on C-5.

Irradiation of H-3 simplified the 2.496 ppm signal to a doublet, thus confirming the assignment. The configuration of the new 5-hydroxyl group was β because H-4α was still clearly deshielded, and the small displacement it suffered correlates well with that of axial protons vicinal to axial hydroxyl groups (25). The significant shift observed on H-3 (0.46 ppm) is similar to the one observed in a model compound for the introduction of a hydroxyl group 1,3-diaxial to the observed proton (26), thus confirming the structure assignment. The pattern of modifications observed in this case is similar to the one found in the case of 5β-hydroxylation of norCDCA (27).

Fig. 5 shows the mass spectrum of the trihydroxy metabolite. The major fragment ions support the structural determination made by the PMR analysis. The fragment ion *m/z* 533 arises by the loss of (90+15) [TMSOH (90)+-CH_3(15)] from the molecular ion. The fragment ion *m/z* 458, the base peak, indicates the loss of two -OTMS groups. The ion at *m/z* 345 arises by cleavage of the C-2,3, C-7,8, and C-5,11 bonds, suggesting the presence of three trimethylsiloxy groups between C-3 and C-7.

TABLE 3. PMR spectrum of 7-Me-norCDCA and its polar metabolite

	7-Me-norCDCA	Polar Metabolite
Me-18	0.722 (s)	0.718 (s)
Me-19	0.872 (s)	0.843 (s)
Me-21	0.994 (d)	0.998 (d)
H-3β	3.493 (m)	3.950 (sep)
H-4α	2.179 (q)J = 12Hz	2.486 (dd)J_1 = 11.6Hz, J_2 = 13.7Hz
H-22	2.434 (dd)J_1 = 2.9Hz, J_1 = 14.3Hz	2.431 (dd)J_1 = 3.2Hz, J_2 = 14.4Hz
Me-7β	1.225 (s)	1.256 (s)

These samples were analyzed by PMR as the methyl esters after dissolution in $CDCl_3$.

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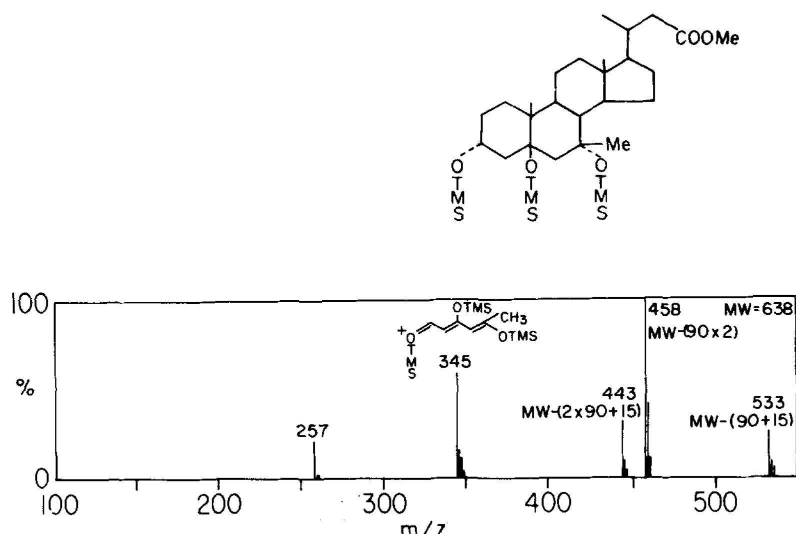

Fig. 5. Mass spectrum of the hydroxy metabolite of 7-Me-norCDCA. The metabolite was analyzed by GLC-MS (column SP-2250) as the methyl ester-TMS ether.

Conjugation pattern

Fig. 6 shows the result of radio-TLC of the bile acid mixture from a bile sample after enzymatic hydrolysis and solvolysis. The number of radioactive spots decreased to two and the R_f values were identical to those of 7-Me-norCDCA and 5β-hydroxy-7-Me-norCDCA (solvent systems A and B). When the enzymatic hydrolysis was performed with a β-glucuronidase from *Helix pomatia* (which also contains a sulfatase), the sulfated bile acid was not converted to the free acid. Since the *Helix pomatia* sulfatase has been reported to hydrolyze steroidal C-3 sulfates rapidly (28), it seems possible that the sulfate group was present at C-7, as is known to occur in the hamster (29). In agreement with this conclusion is the observation that after solvolysis, the R_f changed from that of a sulfated compound to that of 7-Me-norCDCA.

Table 4 shows the biliary bile acid composition after intravenous infusion of 7-Me-norCDCA. Shortly after the infusion period, the parent compound and its hydroxy derivative became the major biliary bile acid constituents. The data obtained following intraduodenal injection were quite similar and are not presented. Evidently, 7-Me-norCDCA was absorbed from the intestine and rapidly secreted into bile. Since its biotransformation was identical to that occurring when this compound was given intravenously, biotransformation was likely to occur in the liver and not in the intestine.

Choleretic effect of 7-Me-norCDCA

Fig. 7 shows bile flow and secretion of radioactivity after intravenous infusion of 7-Me-norCDCA for 120 min. Bile flow increased rapidly at the start of the infusion, and decreased when the infusion was stopped. The increase in bile volume was two or three times that of the saline controls. The rate of secretion of radioactivity into bile paralleled the rate of bile flow. The increment in bile flow (Δμl/μmol · kg), that is, the observed value for bile flow minus the preinfusion (basal) value, was divided by the recovery of radioactivity (Δμmol/kg), to give the apparent choleretic activity (11) of Me-norCDCA and its metabolites; the value ranged from 130 to 200 μl/μmol, a value similar to that reported for nor CDCA in the hamster (11). Most natural C_{24} unconjugated bile acids, when given at

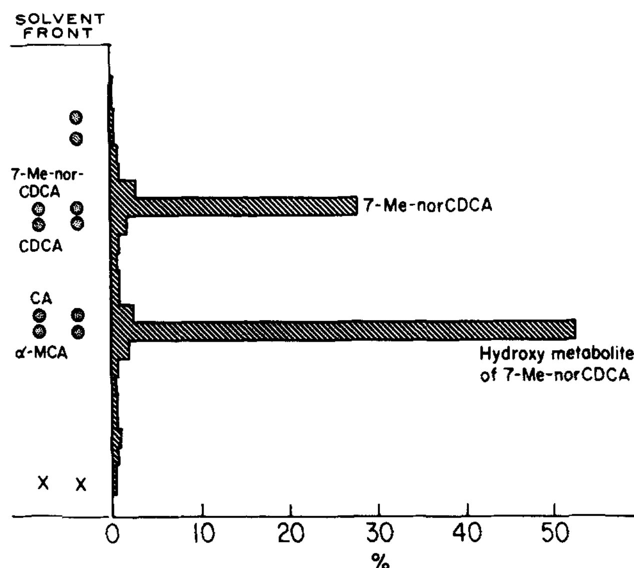

Fig. 6. Thin-layer chromatographic analysis of radioactivity from a hydrolyzed and solvolyzed bile sample of a bile fistula hamster infused iv with [14C]7-Me-norCDCA. The bile sample was analyzed by solvent system B. Reference compounds are as follows: CA, cholic acid; α-MCA, α-muricholic acid; 7-Me-norCDCA, 7-methyl-norchenodeoxycholic acid; CDCA, chenodeoxycholic acid.

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TABLE 4. Biliary bile acid composition of bile fistula hamsters infused intravenously with labeled 7-Me-norCDCA

Bile Acid Infused	Collection Time	Bile Acid Composition				
		CA	CDCA	DCA	7-Me-norCDCA	5β-hydroxy-7-Me-norCDCA
	min			%		
Control	0–20	64.9 ± 10.6	31.8 ± 10.1	3.4 ± 0.5		
	20–40	65.7 ± 12.9	30.9 ± 11.1	3.5 ± 1.9		
	40–60	67.0 ± 14.1	28.7 ± 13.2	4.4 ± 1.0		
	60–80	71.5 ± 10.5	25.1 ± 9.0	3.5 ± 1.5		
	80–100	72.9 ± 8.5	22.9 ± 7.3	4.3 ± 1.6		
	100–120	74.0 ± 9.4	17.1 ± 6.0	6.7 ± 4.6		
	120–140	80.6 ± 10.0	15.2 ± 8.6	4.3 ± 1.4		
	140–160	67.0 ± 0.9	25.3 ± 8.5	7.7 ± 7.6		
	160–180	68.5 ± 1.1	25.1 ± 2.2	6.5 ± 1.1		
	180–200	65.5 ± 3.7	32.5 ± 5.7	2.1 ± 2.0		
	200–220	68.1 ± 2.4	25.9 ± 3.7	6.1 ± 6.0		
	220–240	63.8 ± 8.4	30.0 ± 2.2	6.3 ± 6.2		
	240–260	56.3 ± 5.5	37.0 ± 5.7	6.8 ± 0.2		
7-Me-norCDCA	0–20	60.2 ± 6.6	34.5 ± 4.3	4.8 ± 4.3		
	20–40	62.7 ± 3.9	35.8 ± 3.1	1.5 ± 0.9		
	40–60	55.4 ± 8.2	31.4 ± 1.6	0.8 ± 0.7	2.6 ± 0.8	9.8 ± 6.3
	60–80	29.8 ± 4.8	16.7 ± 1.7	0.6 ± 0.4	13.0 ± 7.9	39.9 ± 9.0
	80–100	40.6 ± 6.3	25.6 ± 9.0	0.5 ± 0.4	6.0 ± 1.1	27.3 ± 16.0
	100–120	59.4 ± 3.4	32.0 ± 4.4	2.4 ± 1.9	1.3 ± 0.1	4.9 ± 3.0
	120–140	62.0 ± 2.5	27.9 ± 5.8	0.9 ± 0.8	1.2 ± 0.4	8.0 ± 7.9
	140–160	61.1 ± 4.7	28.0 ± 7.0	2.7 ± 2.0	1.7 ± 0.4	6.5 ± 6.4
	160–180	69.7 ± 4.7	28.2 ± 3.9	1.5 ± 1.6		0.6 ± 0.5
	180–200	58.1 ± 12.4	37.0 ± 14.3	5.0 ± 2.3		
	200–220	54.5 ± 3.0	42.1 ± 6.4	2.3 ± 2.2		
	220–240	71.1 ± 17.0	28.9 ± 17.0			
	240–260	59.0 ± 0.4	41.0 ± 4.0			

After a control period (time 0–40 min), the ^{14}C-labeled bile acid analogue was infused at a dose of 50 μg/min for 20 min. In the control animals, saline was infused throughout the experiment. The bile acids were extracted with Sep-Pak C_{18} cartridges after enzymatic hydrolysis and were then extracted with ethyl acetate after solvolysis and alkaline hydrolysis. They were analyzed by GLC as methyl ester TMS derivatives. Three animals were studied in each group.

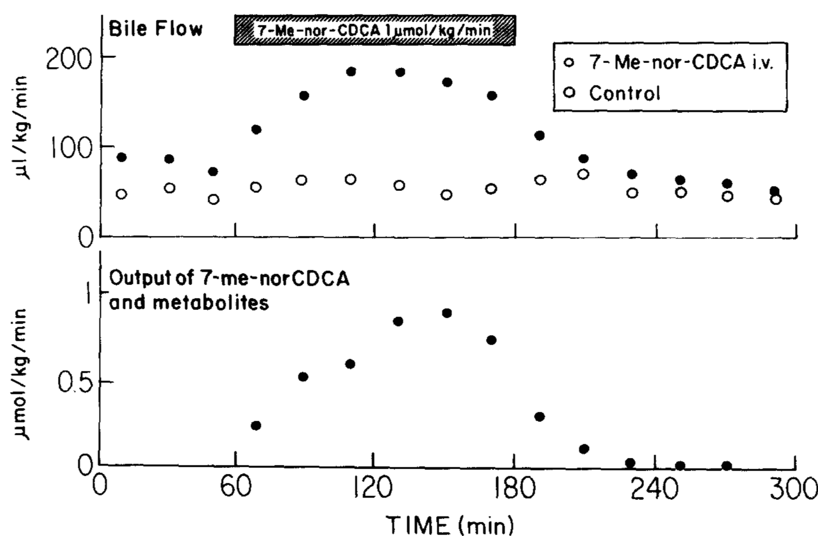

Fig. 7. Bile flow and radioactivity secreted into the bile of bile fistula hamster infused iv with [^{14}C]7-Me-norCDCA. After a 60-min control period, the bile acid was infused at a rate of 1 μmol/min · kg for 2 h. In control animals, saline was infused throughout the experiment; (○), control; (●), 7-Me-norCDCA.

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this dose in the rodent, have an apparent choleretic value of 5–25 μl/μmol (11).

DISCUSSION

Organic synthesis

7-Me-norCDCA was prepared starting from norUDCA methyl ester. The 3-hemisuccinate of methyl norursodeoxycholate was synthesized first in order to protect the hydroxyl group at C-3. CrO_3 oxidation then gave the 7-ketone in good yield. When the starting material was norCDCA methyl ester, it was found that protection of the 3-hydroxyl group was not needed. From the 7-ketone, 7-Me-norCDCA was made by a direct Grignard reaction; protection of the carboxyl group by forming the intermediate oxazoline was unnecessary. However, under the conditions used, only the 7β-methyl-7α-hydroxy-bile acid analogue was formed; the direct Grignard reaction gave 7-Me-norCDCA as the major product (72.6%) and no 7α-methyl-7β-hydroxy bile acid was detected. The predominant formation of the 7β-methyl epimer during the Grignard reaction is ascribed to the bending of the A-ring that shields the α-side of the norbile acid molecule. As a consequence, the Grignard reagent approaches the 7-ketone predominantly from the β-side (30). It seems likely that synthesis of the 7α-methyl epimer will require formation of the 7-oxo-oxazoline intermediate. Reaction of the latter with CH_3MgI followed by mild hydrolysis may then result in a mixture of 7-Me-norCDCA and 7-Me-norUDCA, which requires chromatography for separation of the two epimers.

Hepatic biotransformation to a new trihydroxy bile acid

The hepatic metabolism of 7-Me-norCDCA in the hamster resembled that of norCDCA both qualitatively and quantitatively (11, 27). Following the iv injection of labeled 7-Me-norCDCA, most of the radioactivity secreted into the bile was in the form of a novel trihydroxy bile acid whose structure was identified as 5β-hydroxy-7-Me-norCDCA (3α,5β,7α-trihydroxy-7β-methyl-24-nor-5β-cholan-23-oic acid). Previously, it had been found that in the hamster, after iv infusion, norCDCA was also converted in the liver to its 5β-hydroxy derivative (27). In the present study, although the polar metabolite of 7-Me-norCDCA resembled a 3,6,7-trihydroxy acid because of its R_f value on TLC, the PMR data showed that this was not the case. (If the unknown were hydroxylated at C-6, the H-3β signal should not move and a secondary alcohol signal should appear. Further, for a 6β-hydroxyl group, the 19-Me signal should shift to 1.5 ppm.) The spin combination of H-4α, the shifts of the H-3β and H-4α signals, and the absence of a signal of a new secondary alcohol in comparison with 7-Me-norCDCA indicate that the proton at H-5β had disappeared with the formation of a tertiary alcohol.

Conjugation

In previous experiments, the metabolic fate of the C_{24} 6-Me and 7-Me bile acids was found to be similar to that of the naturally occurring C_{24} bile acids, CA, CDCA, HDCA, and MDCA (3–5), that is, the methyl analogues were conjugated efficiently with taurine or glycine and were excreted into the bile (3–5). During enterohepatic cycling, they were not dehydroxylated in either the 6- or 7-position, i.e., no methyl-substituted lithocholate analogues were detectable in bile or feces (3–5).

In the present experiment, there was little amidation of 7-Me-norCDCA with taurine (13.7 ± 5.0%) and no amidation with glycine. In the case of norCDCA, amidation with taurine was similarly low and amidation with glycine was not observed (11). Other C_{23} bile acids are not readily conjugated with glycine or taurine in the liver (9–15). The limited amidation is ascribed to the fact that C_{23} norbile acids appear to be poor substrates for the two key enzymes, bile acid-CoA ligase (31) and cholyl-CoA glycine/taurine-N-acyltransferase (32).

The biotransformation of 7-Me-norCDCA in the hamster differed little from that of norCDCA. With both norCDCA and 7-Me-norCDCA, the free acid was secreted in the bile to a very limited extent (about 10% of biliary radioactivity). Both norbile acids were hydroxylated at the 5β-position, about 40% of the biliary radioactivity in the case of norCDCA and as much as 50% with 7-Me-norCDCA. This relatively small difference may be due to the greater hydrophobicity of the 7-methyl analogue. Since 7-Me-norCDCA is more hydrophobic than norCDCA (upon reversed phase HPLC the relative retention time of 7-Me-norCDCA is 1.3 times greater than that of norCDCA), we speculate that the unchanged form partitions to a greater extent into the endoplasmic reticulum where it undergoes hydroxylation or glucuronidation.

In the case of the two C_{23} bile acids, norCDCA and 7-Me-norCDCA, 5β-hydroxylation was unexpected. It is known that the naturally occurring C_{24} bile acids can be hydroxylated at many positions, both on the nucleus and in the side chain, but C-5 hydroxylation has not been reported previously (33). The introduction of a novel nuclear hydroxyl group has been attributed to the lack of CoA ester formation. As a consequence, such bile acids regurgitate from the liver cell into the sinusoids and undergo reuptake by the pericentral hepatocytes, exposing them to a new gruop of microsomal enzymes (34).

Only a single experiment was performed with CDCA because the hepatic biotransformation of this compound has been rather well characterized in previous experiments in the rat (35, 36), baboon (37), rabbit (38), hamster (11, 12), and humans (39). In all of these mammals, CDCA is efficiently conjugated with glycine or taurine, and little conjugation with sulfate or glucuronate occurs. There is limited hydroxylation in some species at the 6 position and there may also be oxido reduction at the 7 posi-

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tion to form β-muricholic acid (35, 37). Hydroxylation at C_5 has been reported previously for UDCA in the isolated perfused rat liver (18), but the proportion formed was quite small.

Choleresis

When 7-Me-norCDCA was infused intravenously, bile flow was increased 2- to 3-fold. The apparent choleretic activity of 7-Me-norCDCA (and its metabolites) (130–200 μl/μmol) indicates that hypercholeresis was induced. This effect of the 7-methyl-nor acid is similar to that produced by other dihydroxy norbile acids, such as norCDCA (11) or norUDCA (10, 12), and high doses of UDCA (40, 41), hyodeoxycholic acid (42), or 12-epi-deoxycholic acid (43). The most likely cause of the hypercholeresis is cholehepatic shunting as proposed for other hypercholoretic dihydroxy nor bile acids (9–12, 16–19). An intrahepatocyte stimulatory effect of the norbile acid analogue causing secretion of bicarbonate into the canaliculus, as has been proposed for the hypercholeretic effect of UDCA (44, 45), cannot be excluded. A similar choleresis was observed when 7-Me-norCDCA was given intraduodenally.

The extent to which 7-Me-norCDCA and its metabolites undergo enterohepatic cycling was not defined by these experiments. 7-Me-norCDCA, similar to other unconjugated dihydroxy bile acids (39, 45–48), was rapidly and passively absorbed from the small intestine. The unconjugated trihydroxy metabolite formed in the liver is also likely to be absorbed passively from the small intestine, albeit more slowly (16, 46, 47). Whether this trihydroxy compound will remain in the enterohepatic circulation after chronic feeding experiments is unknown since the metabolism of this compound has not been defined. The glucuronate and sulfate conjugates of 7-Me-norCDCA that are formed in the liver are unlikely to be absorbed from the small intestine unless they undergo deconjugation. The taurine conjugate that is formed to a very limited extent will be reabsorbed efficiently from the ileum, but some will pass into the colon where deconjugation is likely to occur, based on experiments with the taurine conjugate of nor-7β-cholate in the rat (49). If the liberated unconjugated 7-Me-norCDCA is then reabsorbed from the colon, it will undergo the biotransformation events of the parent compound. The net result of these multiple biotransformation pathways should be that 7-Me-norCDCA is likely to be rapidly lost from the enterohepatic circulation. In agreement with this prediction is the experimental observation that, in the rabbit, chronically administered norUDCA does not accumulate appreciably in biliary bile acids (50).

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